

# p53 Induction as a Genotoxic Test for Twenty-Five Chemicals Undergoing *In Vivo* Carcinogenicity Testing

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*In vivo* carcinogenicity testing is an expensive and time-consuming process, and as a result, only a relatively small fraction of new and existing chemicals has been tested in this manner. Therefore, the development and validation of alternative approaches is desirable. We previously developed a mammalian *in vitro* assay for genotoxicity based on the ability of cells to increase their level of the tumor-suppressor protein p53 in response to DNA damage. Cultured cells are treated with various amounts of the test substances, and at defined times following treatment, they are harvested and lysed. The lysates are analyzed for p53 by Western blot and/or enzyme-linked immunosorbent assay analysis. An increase in cellular p53 following treatment is interpreted as evidence for DNA damage. To determine the ability of this p53-induction assay to predict carcinogenicity in rodents and to compare such results with those obtained using alternate approaches, we subjected 25 chemicals from the predictive toxicology evaluation 2 list to analysis with this method. Five substances (citral, cobalt sulfate heptahydrate, D&C Yellow No. 11, oxymetholone, and *t*-butylhydroquinone) tested positive in this assay, and three substances (emodin, phenolphthalein, and sodium xylenesulfonate) tested as possibly positive. Comparisons between the results obtained with this assay and those obtained with the *in vivo* protocol, the *Salmonella* assay, and the Syrian hamster embryo (SHE) cell assay indicate that the p53-induction assay is an excellent predictor of the limited number of genotoxic carcinogens in this set, and that its accuracy is roughly equivalent to or better than the *Salmonella* and SHE assays for the complete set of chemicals. **Key words:** assay development, carcinogenicity, genotoxicity, p53, PTE2. *Environ Health Perspect* 107:805–812 (1999). [Online 1 September 1999]  
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Because society has made human health and safety an important consideration, considerable resources have been and are being expended in efforts to identify and classify human carcinogens. Unfortunately, methods currently used, such as the Ames test (1) and *in vivo* animal testing, suffer from several shortcomings. These include the need to extrapolate from prokaryotes to humans (Ames test) and the need to extrapolate from rodents to humans, the high cost, and the long period before results are known (*in vivo* animal testing). With all current methods there is also limited predictivity, both when examining the agreement of various methods with each other (2–6) and when applying laboratory results to actual human populations.

To aid in the development and validation of improved ways to predict the carcinogenicity of compounds, the National Institute of Environmental Health Sciences (NIEHS; Research Triangle Park, NC) initiated the predictive toxicology evaluation project (PTE). This project listed two sets of chemicals undergoing *in vivo* carcinogenicity testing; the first set of 44 chemicals was listed in 1990 (PTE1) and the second set of 30 chemicals in 1994 (PTE2) (7,8). Researchers were invited to submit predictions regarding the ability of these chemicals to induce carcinogenesis in rodents, and the predictions and actual *in vivo* results were to be compared when the animal results

became available. The results from PTE1 provided information regarding the features of chemicals most predictive of their ability to act as carcinogens, and the results from PTE2 will add to this dataset once all of the *in vivo* results are available for comparison with the predictions.

We previously developed a novel method for assessing the genotoxicity of substances that is simple, rapid, and cost-effective. This method is based on the biologic response of mammalian cells in culture to agents that damage their DNA (9). This assay is based on the well-documented observation that the cellular level of a tumor-suppressor protein called p53 increases following DNA damage (10–15), primarily because of an increase in the stability of the protein and a resulting decrease in its rate of proteolysis (9,10). The p53 then acts to prevent replication of damaged DNA, either by causing the cell to undergo a reversible growth arrest (11,16,17) or by initiating a cell's apoptotic pathway (18–21).

The assay itself is simple (9). Cultured mammalian cells (NCTC 929) are treated with various doses of the test agent, and at specified time points following treatment, the cells are harvested and lysed. The level of p53 in the lysates is measured by p53 enzyme-linked immunosorbent assay (ELISA) and/or Western blot analysis, and compared to the level in untreated cells.

Our previous work (9) indicated that both Western blot and ELISA analyses yielded similar results. Therefore, our current work focused on the more quantitative ELISA assay. We also found that the NCTC 929 cell line was a useful model system. NCTC 929 cells were initially selected because previously published results (10) indicated that their levels of p53 rise following genotoxin treatment. We verified this, and found in addition that p53 in these cells can be immunoprecipitated by a monoclonal antibody specific for wild-type p53. We also found that the level of p53 in these cells rises significantly following treatment with indirect genotoxins such as aflatoxin B<sub>1</sub> and 2-acetylaminofluorene, indicating that they possess significant amounts of the metabolic activities necessary for biotransformation (9).

To determine the ability of this assay to predict carcinogenicity in rodents and to compare its performance with other proposed alternatives, we subjected 25 of the 30 PTE2 substances to analysis by this method. Five substances (citral, cobalt sulfate heptahydrate, D&C Yellow No. 11, oxymetholone, and *t*-butylhydroquinone) tested positive in this assay and three substances (emodin, phenolphthalein, and sodium xylenesulfonate) tested as possibly positive. Comparisons between the results obtained with this assay and those obtained with the *in vivo* protocol, the *Salmonella* assay, and the Syrian hamster embryo (SHE) cells indicate that the p53-induction assay is an excellent predictor of the limited number of genotoxic carcinogens in this set, and that its accuracy is roughly equivalent to or better than the *Salmonella* and SHE assays for the complete set of chemicals.

## Materials and Methods

**Cell culture.** These experiments were done using the NCTC 929 cell line derived from mouse fibroblasts and obtained from the American Type Culture Collection

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(Rockville, MD). Cells were cultured in modified Eagle media (Gibco/Life Sciences, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco) (MEM 10). Cells were plated at a density of  $0.5\text{--}1.5 \times 10^6$  cells per 100 mm plate 1 day prior to treatment. pAB 122 hybridoma cells, also obtained from the American Type Culture Collection, were propagated in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, and served as the source for the primary anti-p53 antibody used in the ELISA analysis.

**Chemicals.** Fourteen of the 25 substances tested were obtained from the NIEHS. These substances and their Chemical Abstract Service (CAS) numbers are as follows: codeine (76-57-3); D&C Yellow No. 11 (8003-22-3); diethanolamine (111-42-2); 1,2-dihydro-2,2,4-trimethylquinoline (147-47-7); ethylbenzene (100-41-4); ethylene glycol monobutyl ether (111-76-2); furfuryl alcohol (98-00-0); gallium arsenide (1303-00-0); methyleugenol (93-15-2); oxymetholone (434-07-1); phenolphthalein (77-09-8); primaclone (125-33-7); pyridine (110-86-1); and scopolamine hydrobromide trihydrate (6533-68-2). Ten substances were obtained from Sigma/Aldrich (St. Louis, MO): 1-chloro-2-propanol (127-00-4), citral (5392-40-5), cobalt sulfate heptahydrate (10026-24-1), emodin (518-82-1), isobutyraldehyde (78-84-2), nitromethane (75-52-5), sodium nitrate (7632-00-0), sodium xylensulfonate (1300-72-7), *t*-butylhydroquinone (1948-33-0), and tetrahydrofuran (109-99-9). Cinnamaldehyde (104-55-2) was obtained from Acros (Pittsburgh, PA).

Unless noted otherwise, 10 mg/mL stock solutions of the test substances were prepared in either culture media (MEM 10) or in dimethylsulfoxide (DMSO). We have previously shown that DMSO alone at the concentrations used does not induce p53 in treated cells (9).

**Cell treatment.** Volumes of the stock solution corresponding to the doses tested were added to a series of plates (approximately  $1 \times 10^6$  cells per 100 mm plate in a volume of 10-mL MEM 10). Control plates were treated with an amount of vehicle alone (media or DMSO) that corresponded to the highest volume added to the experimental plates. For each chemical, several doses between 1 and 100  $\mu\text{g/mL}$  of the test substance were evaluated; the exact doses used are listed in the sections for the separate chemicals. Treated and control cells were incubated at 37°C and 5%  $\text{CO}_2$  until harvest. One series was harvested approximately 6 hr posttreatment, and a second series was harvested approximately 17 hr posttreatment. Each chemical was tested, at the several dosages and at the two time points, between

two and four times. In some cases, specific doses were repeated in additional trials.

To ensure that the p53 response of these cells to both direct- and indirect-acting genotoxic chemicals remained intact and relatively constant during these experiments, cultures of NCTC 929 cells from passages currently in use were periodically treated with known direct (*N*-methyl-*N'*-nitro-nitrosoguanidine) and indirect (mitomycin C) genotoxins (9) to verify that the cells could adequately respond to both types of chemicals. We found that the cellular response to both direct- and indirect-acting chemicals was stable throughout and beyond the course of these experiments.

**Cell lysis and p53 ELISA.** Cells were harvested, lysed, and analyzed for their protein concentration by the BCA assay (Pierce, Rockford, IL) and for their p53 levels by ELISA, as described previously (9). Briefly, cells were removed from the plate by trypsinization, concentrated by centrifugation, and suspended in lysis buffer. After lysis, lysates were stored at -80°C for no more than 1 week prior to analysis. The ELISA analysis used pAB 122 as the primary or capture antibody, biotinylated anti-p53 (Boehringer Mannheim, Annapolis, IN) as the detection antibody, and GST-p53 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as a standard. Each point was measured in triplicate, and the results were normalized to the amount of protein present in each sample.

**Analysis.** Data were analyzed using one-way analysis of variance software (SuperAnova, Abacus Concepts, Berkeley, CA). The Scheffe *S*-test was used post hoc to identify significant differences between p53 levels in treated and untreated cells. Test results were interpreted as positive if one or more doses yielded a significant increase ( $p \leq 0.001$ ) in the level of p53 in treated versus untreated cells, in two or more separate experiments, at one or both

of the times tested (6 and 17 hr), and with no more than one of three or four experiments failing to show such an increase. Results were interpreted as possibly positive using the same criteria, except that the significance of the increase was less ( $p \leq 0.05$ ).

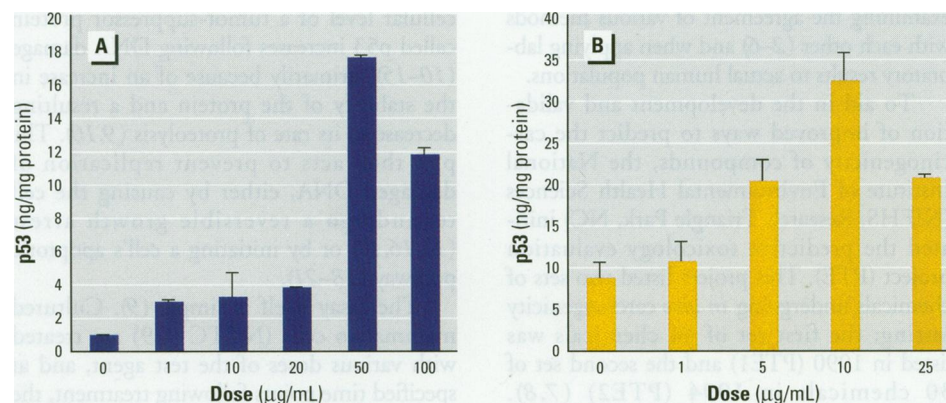
Data regarding the *Salmonella* and *in vivo* results (where available) were obtained from the PTE2 website (8).

## Results

### *Cobalt sulfate heptahydrate and D&C Yellow No. 11 induce p53 strongly by 6 hr.*

Two substances, cobalt sulfate heptahydrate and D&C Yellow No. 11, caused increases in cellular p53 by 6 hr. The increases for cobalt sulfate heptahydrate were significant at both 50 and 100  $\mu\text{g/mL}$ , and those for D&C Yellow No. 11 were significant at 5, 10, and 25  $\mu\text{g/mL}$ . On the basis of these results, they are classified as positive. Figure 1 depicts representative experiments for cells treated with each of these substances and harvested at 6 hr. For these two substances, the increase in the cellular level of p53 in cells treated with the statistically significant doses is robust—greater than a 4-fold increase over that seen in untreated cells.

*Citral, cobalt sulfate heptahydrate, D&C Yellow No. 11, oxymetholone, and t-butylhydroquinone induce p53 strongly by 17 hr.* In addition to the two substances that induced p53 by 6 hr, three additional substances caused significant increases in the cellular level of p53 by 17 hr (citral, oxymetholone, and *t*-butylhydroquinone). The increase for citral was significant at doses of 10, 15, 20, 25, and 30  $\mu\text{g/mL}$ ; some cytotoxicity was noted at 25 and 30  $\mu\text{g/mL}$  and increased cytotoxicity at 50  $\mu\text{g/mL}$ . The increases for cobalt sulfate heptahydrate were significant at doses of 20 and 50  $\mu\text{g/mL}$ ; significant cytotoxicity was noted in cells treated with 100  $\mu\text{g/mL}$  for 17 hr. Cells treated with 25



**Figure 1.** Chemicals that strongly increased cellular p53 levels by 6 hr. (A) Cobalt sulfate heptahydrate. (B) D&C Yellow No. 11. Cultured mammalian (NCTC 929) cells were treated with the indicated doses of the test substances, then harvested and lysed at 6 hr posttreatment. The level of p53 present in the lysates was quantitated by enzyme-linked immunosorbent assay, as described in "Materials and Methods." Error bars represent standard error.



$\mu\text{g/mL}$  D&C Yellow No. 11 for 17 hr showed a significant increase in their cellular p53 level; cells treated with a higher level displayed cytotoxicity. Oxymetholone caused significant increases in the p53 level at doses of 10 and 25  $\mu\text{g/mL}$ , with increasing cytotoxicity noted at doses of 25 and 50  $\mu\text{g/mL}$ . Finally, *t*-butylhydroquinone significantly increased cellular p53 levels at doses of 1, 5, and 10  $\mu\text{g/mL}$ , with significant cytotoxicity noted at higher doses. On the basis of these results, these five substances are classified as positive. Figure 2 depicts representative experiments for cells treated with each of these substances and harvested at 17 hr. For each of the substances that tested positive, the increase in the cellular level of p53 in cells treated with the statistically significant doses is robust—greater than a 4-fold increase over that seen in untreated cells.

**Emodin, phenolphthalein, and sodium xylenesulfonate induce p53 less strongly by 17 hr.** Emodin increased cellular p53 levels at a dose of 20  $\mu\text{g/mL}$ , phenolphthalein at a dose of 10  $\mu\text{g/mL}$  by 17 hr, and sodium xylenesulfonate at doses of 1 and 5  $\mu\text{g/mL}$ . In the case of these three chemicals, the significance of the increase was less than that noted for the five chemicals previously discussed; hence, they are classified as possible positives. Figure 3 depicts representative experiments for cells treated with each of these substances and harvested at 17 hr. The extent of the increase in p53 was reduced (approximately 2- to 3-fold over that seen in untreated cells) as compared to that of the previous five substances.

**Seventeen chemicals did not increase cellular levels of p53.** The remaining seventeen chemicals (1-chloro-2-propanol, cinnamaldehyde, codeine, diethanolamine,

1,2-dihydro-2,2,4-trimethylquinoline, ethylbenzene, ethylene glycol monobutyl ether, furfuryl alcohol, gallium arsenide, isobutyraldehyde, methyleugenol, nitromethane, primaclone, pyridine, scopolamine hydrobromide trihydrate, sodium nitrite, and tetrahydrofuran) were unable to reproducibly and significantly increase cellular p53 levels at the times and doses tested. Based on these results, they are classified as negative.

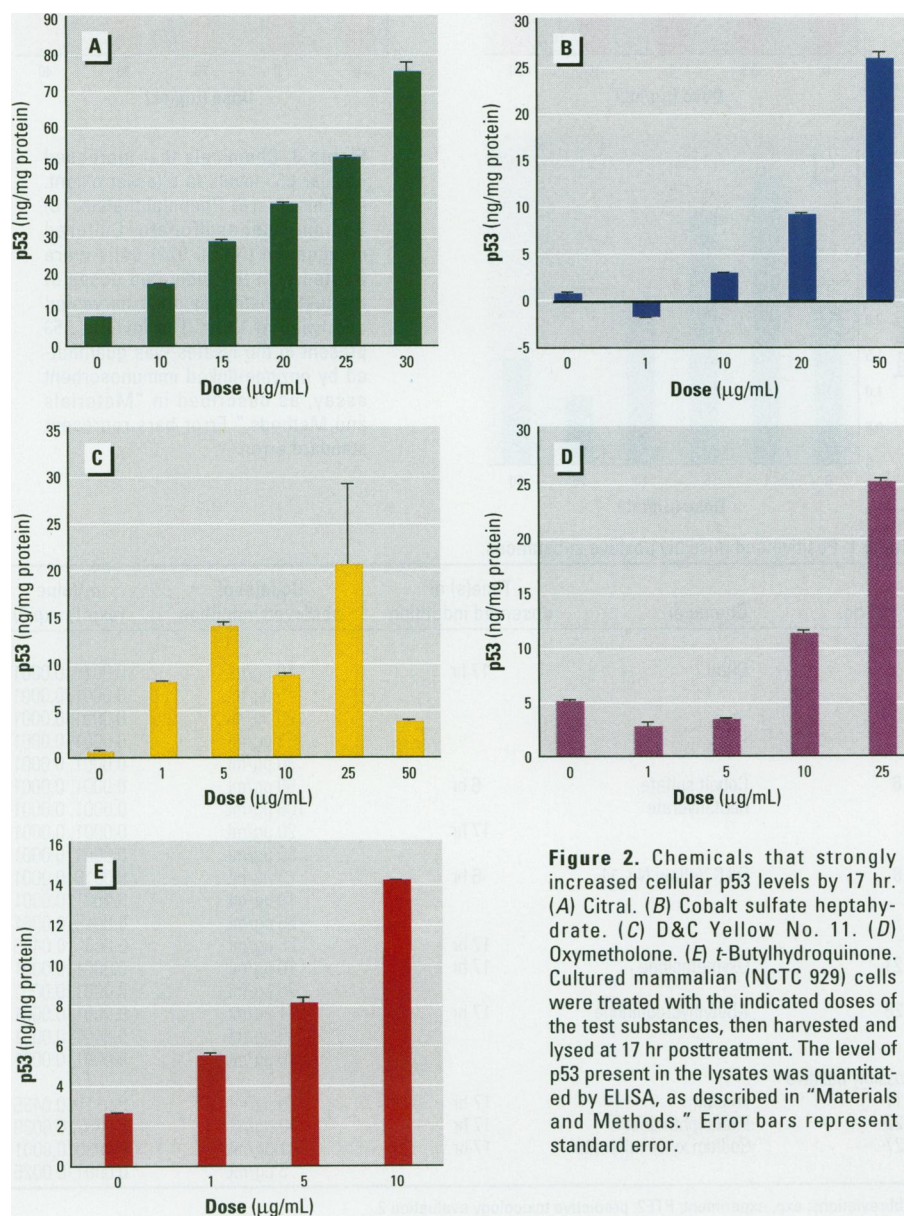
**Substances classified on the basis of their ability to increase cellular p53.** Table 1 lists the substances that tested positive ( $p \leq 0.001$ ) or possibly positive ( $p \leq 0.05$ ) in this assay, along with the time(s) and dose(s) at which positive results were achieved. Five substances (citral, cobalt sulfate heptahydrate, D&C Yellow No. 11, oxymetholone, and *t*-butylhydroquinone) gave clearly positive signals. Cobalt sulfate heptahydrate and D&C Yellow No. 11 were positive at both the early and late time points, whereas the remaining three were positive only at the later time point. Three substances (emodin, phenolphthalein and sodium xylenesulfonate) were possibly positive in our assay. That is, they demonstrated the ability to reproducibly induce p53 at at least one tested dose, although the increase was less significant than seen with the previous five substances.

**Summary.** Table 2 shows the summary data for the substances that we tested as well as previously reported results from the *Salmonella* and SHE assays (22). It also lists a summary of the *in vivo* test results where available. In this table, the either- or single-species *in vivo* test results are listed as positive if there is some or clear evidence of carcinogenicity in any of the four treatment groups; the transspecies results are listed as positive only if there was some or clear evidence of carcinogenicity in each of the two tested species.

Specific notes regarding the individual substances tested follow. Classifications (positive, possibly positive, and negative) refer to the results of this study.

**1-Chloro-2-propanol.** A 10-mg/mL stock of 1-chloro-2-propanol was prepared in DMSO, and cells were treated with doses of 1, 5, 10, 25, and 50  $\mu\text{g/mL}$ . Cells treated with 1-chloro-2-propanol did not display cytotoxicity at any of the times or doses tested, and 1-chloro-2-propanol did not induce p53 at any of the doses or times tested. 1-Chloro-2-propanol is therefore classified as negative by this method; this contrasts with positive results in the *Salmonella* test and the SHE assay. The *in vivo* test results were also negative.

**Cinnamaldehyde.** A 10-mg/mL stock of cinnamaldehyde was prepared in DMSO, and cells were treated with doses of 1, 10, 20, and 50  $\mu\text{g/mL}$ . Cells treated with cinnamaldehyde displayed cytotoxicity at the highest dose



**Figure 2.** Chemicals that strongly increased cellular p53 levels by 17 hr. (A) Citral. (B) Cobalt sulfate heptahydrate. (C) D&C Yellow No. 11. (D) Oxymetholone. (E) *t*-Butylhydroquinone. Cultured mammalian (NCTC 929) cells were treated with the indicated doses of the test substances, then harvested and lysed at 17 hr posttreatment. The level of p53 present in the lysates was quantitated by ELISA, as described in "Materials and Methods." Error bars represent standard error.



tested (50 µg/mL) at both 6 and 17 hr. Cinnamaldehyde did not induce p53 at any of the doses or times tested, and is therefore classified as negative. The *Salmonella* results were weakly positive.

**Citral.** A 10-mg/mL stock of citral was prepared in DMSO, and cells were treated with doses of 1, 5, 10, 15, 20, 25, 30, and 50 µg/mL. Cells treated with citral displayed increasing cytotoxicity at the highest doses tested (25, 30, and 50 mg/mL) by 6 and 17 hr. Citral induced p53 by 17 hr, and is therefore classified as positive. This contrasts with the results from the *Salmonella* test, which was negative.

**Cobalt sulfate heptahydrate.** A 10-mg/mL stock of cobalt sulfate heptahydrate was prepared in H<sub>2</sub>O, and cells were treated with doses of 1, 10, 20, 50, and 100 µg/mL. Cells treated with cobalt sulfate heptahydrate displayed cytotoxicity when treated with the substance at 100 µg/mL for 17 hr. Cobalt sulfate heptahydrate strongly induced p53 at both the 6 and 17 hr time points, and is therefore classified as positive. The *Salmonella* test gave a weakly positive response, and the SHE assay was also positive. Animal tests have shown some evidence for carcinogenicity in male rats and clear evidence for carcinogenicity in female rats, male mice, and female mice.

**Codeine.** A 10-mg/mL stock of codeine was prepared in DMSO, and cells were treated with doses of 1, 5, 10, 25, and 50 µg/mL. Cells treated with codeine did not display cytotoxicity at any of the times or doses tested, and codeine did not induce p53 at any of the doses or times tested. Codeine is therefore classified as negative. This is in agreement with the negative test results seen in the *Salmonella* and SHE assays, and a lack of evidence for carcinogenicity seen in the animal tests.

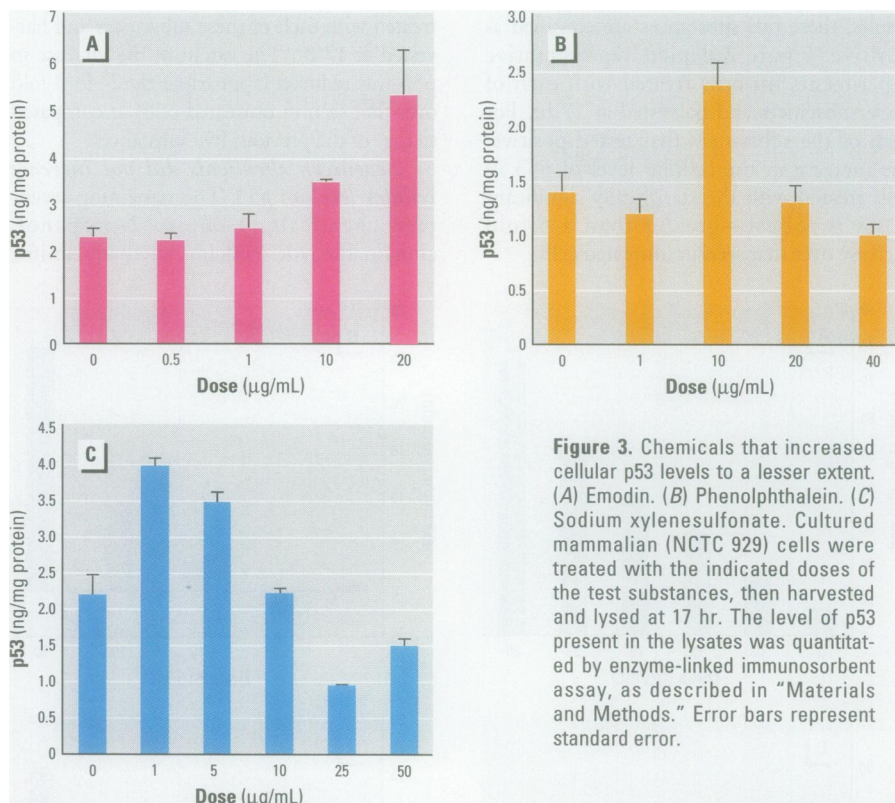
**D&C Yellow No. 11.** A 5-mg/mL stock of D&C Yellow No. 11 was prepared in DMSO, and cells were treated with doses of 1, 5, 10, 25, and 50 µg/mL. Cells treated with D&C Yellow No. 11 displayed some cytotoxicity at 50 µg/mL by 17 hr. The actual doses received by the cells were less than those administered, as some precipitation of the substance was observed after its addition to the media. Cells took up the dye; cell pellets were yellow. This color partitioned with the membrane fraction during centrifugation of the lysate. D&C Yellow No. 11 strongly induced p53 at both 6 and 17 hr, and is therefore classified as positive. The *Salmonella* test gave a weakly positive result, the SHE test results were positive, and in animal studies, male and female rats showed some evidence of carcinogenicity.

**Diethanolamine.** A 10-mg/mL stock of diethanolamine was prepared in DMSO, and cells were treated with doses of 1, 5, 10,

25, and 50 µg/mL. Cells treated with diethanolamine did not display cytotoxicity at any of the times or doses tested, and diethanolamine did not induce p53 at any of the doses or times tested. Diethanolamine is therefore classified as negative. This is in agreement with a negative result in the *Salmonella* assay, and contrasts with a positive result from the SHE test. During animal testing, no evidence for carcinogenicity was

seen in male or female rats, whereas clear evidence was observed in male and female mice.

**1,2-Dihydro-2,2,4-trimethylquinoline (DTQ).** A 10-mg/mL stock of DTQ was prepared in DMSO, and cells were treated with doses of 1, 5, 10, 25, and 50 µg/mL. Cells treated with DTQ did not display cytotoxicity at any of the times or doses tested, and DTQ did not induce p53 at any of the doses or times tested. DTQ is therefore classified as



**Figure 3.** Chemicals that increased cellular p53 levels to a lesser extent. (A) Emodin. (B) Phenolphthalein. (C) Sodium xylenesulfonate. Cultured mammalian (NCTC 929) cells were treated with the indicated doses of the test substances, then harvested and lysed at 17 hr. The level of p53 present in the lysates was quantitated by enzyme-linked immunosorbent assay, as described in "Materials and Methods." Error bars represent standard error.

**Table 1.** Positive and possibly positive substances.

PTE2 no.	Chemical	Time(s) of observed induction	Dose(s) of observed induction	p-Value (exp 1, exp 2)
Positive				
5	Citral	17 hr	10 µg/mL	0.0019, 0.0001
			15 µg/mL	0.0001, 0.0001
			20 µg/mL	0.0001, 0.0001
			25 µg/mL	0.0001, 0.0001
			30 µg/mL	0.0001, 0.0001
6	Cobalt sulfate heptahydrate	6 hr	50 µg/mL	0.0001, 0.0001
		17 hr	100 µg/mL	0.0001, 0.0001
			20 µg/mL	0.0001, 0.0001
			50 µg/mL	0.0001, 0.0001
8	D&C Yellow No. 11	6 hr	5 µg/mL	0.0016, 0.0001
			10 µg/mL	0.0001, 0.0001
			25 µg/mL	0.0015, 0.0001
21	Oxymetholone	17 hr	25 µg/mL	0.0001, 0.0485
		17 hr	10 µg/mL	0.0001, 0.0001
			25 µg/mL	0.0001, 0.0001
28	t-Butylhydroquinone	17 hr	1 µg/mL	0.0001, 0.0001
			5 µg/mL	0.0001, 0.0001
			10 µg/mL	0.0001, 0.0001
Possibly positive				
11	Emodin	17 hr	20 µg/mL	0.0110, 0.0435
22	Phenolphthalein	17 hr	10 µg/mL	0.0387, 0.0039
27	Sodium xylenesulfonate	17 hr	1 µg/mL	0.0035, 0.0001
			5 µg/mL	0.0001, 0.0025

Abbreviations: exp, experiment; PTE2, predictive toxicology evaluation 2.

negative. This is in agreement with a negative result from the *Salmonella* assay, and contrasts with a positive result from the SHE test. The animal results list some evidence for carcinogenicity in male rats and no evidence in female rats, male mice, and female mice.

**Emodin.** A 10-mg/mL stock of emodin was prepared in DMSO, and cells were treated with doses of 0.5, 1, 10, 20, and 50 µg/mL. Cells treated with emodin displayed some cytotoxicity when treated with the substance at 50 µg/mL for 17 hr. Emodin weakly induced p53 by 17 hr, and is therefore classified as possibly positive. The *Salmonella* and SHE tests gave positive responses.

**Ethylbenzene.** A 10-mg/mL stock of ethylbenzene was prepared in DMSO, and cells were treated with doses of 1, 10, 20, and 50 µg/mL. Cells treated with ethylbenzene did not display significant cytotoxicity at any of the times or doses tested, and ethylbenzene did not induce p53 at any of the doses or times tested. Ethylbenzene is therefore classified as negative. This is in agreement with a negative result from the *Salmonella* assay and contrasts with a positive result from the SHE test. The animal results list clear evidence for carcinogenicity in male rats, and some evidence in female rats, male mice, and female mice.

**Ethylene glycol monobutyl ether (EGMBE).** A 10-mg/mL stock of EGMBE

was prepared in MEM 10, and cells were treated with doses of 1, 5, 10, 25, and 50 µg/mL. Cells treated with EGMBE did not display cytotoxicity at any of the times or doses tested, and EGMBE did not induce p53 at any of the doses or times tested. EGMBE is therefore classified as negative. This is in agreement with a negative result from the *Salmonella* assay and contrasts with a positive result from the SHE assay. The animal results list some evidence in male and female mice, no evidence in male rats, and equivocal evidence in female rats.

**Furfuryl alcohol.** A 10-mg/mL stock of furfuryl alcohol was prepared in MEM 10, and cells were treated with doses of 1, 5, 10, 25, and 50 µg/mL. Cells treated with furfuryl alcohol did not display cytotoxicity at any of the times or doses tested, and furfuryl alcohol did not induce p53 at any of the doses or times tested. Furfuryl alcohol is therefore classified as negative. This is in agreement with negative results from the *Salmonella* and the SHE assays. The animal results list equivocal evidence for carcinogenicity in female rats, some evidence in male rats and mice, and no evidence in female mice.

**Gallium arsenide.** A 1-mg/mL stock of gallium arsenide was prepared in DMSO, and cells were treated with doses of 1, 10, 20, and 50 µg/mL. The actual dose received by the cells is lower than that listed, as some

precipitation could be observed following the addition of the substance to the media. Cells treated with gallium arsenide displayed some cytotoxicity at the highest doses tested (20 and 50 µg/mL), and gallium arsenide did not induce p53 at any of the doses or times tested. Gallium arsenide is therefore classified as negative. This is in agreement with a negative result from the *Salmonella* assay and contrasts with a positive result from the SHE assay.

**Isobutyraldehyde.** A 10-mg/mL stock of isobutyraldehyde was prepared in DMSO, and cells were treated with doses of 1, 10, 20, 50, and 100 µg/mL. Cells treated with isobutyraldehyde did not display cytotoxicity at any of the times or doses tested, and isobutyraldehyde did not induce p53 at any of the doses or times tested. Isobutyraldehyde is therefore classified as negative. The *Salmonella* results were inconclusive, the SHE results were negative, and no evidence for carcinogenicity was observed in male or female rats or in male or female mice.

**Methyleugenol.** A 10-mg/mL stock of methyleugenol was prepared in DMSO, and cells were treated with doses of 1, 5, 10, 25, and 50 µg/mL. Cells treated with methyleugenol did not display cytotoxicity at any of the times or doses tested, and methyleugenol did not induce p53 at any of the doses or times tested. Methyleugenol is therefore classified as negative. This is in agreement with a negative result from the *Salmonella* assay and contrasts with a positive result from the SHE assay. In vivo test results showed clear evidence for carcinogenicity in male and female rats and male and female mice.

**Nitromethane.** A 10-mg/mL stock of nitromethane was prepared in DMSO, and cells were treated with doses of 1, 10, 20, and 50 µg/mL. Cells treated with nitromethane did not display cytotoxicity at any of the times or doses tested, and nitromethane did not induce p53 at any of the doses or times tested. Nitromethane is therefore classified as negative. This is in agreement with a negative result from the *Salmonella* assay, and contrasts with a positive result from the SHE assay. The animal results list no evidence for carcinogenicity in male rats and clear evidence in female rats, male mice, and female mice.

**Oxymetholone.** A 10-mg/mL stock of oxymetholone was prepared in DMSO, and cells were treated with doses of 1, 5, 10, 25, and 50 µg/mL. Cells treated with oxymetholone displayed cytotoxicity at 50 µg/mL by 6 hr and at 25 and 50 µg/mL by 17 hr. Oxymetholone strongly induced p53 by 17 hr, and is therefore classified as positive. This is in contrast to a negative result from the *Salmonella* assay, but in agreement with a positive result from the SHE assay.

**Table 2.** Summary and comparisons.

PTE2 no.	Name	CAS no.	<i>Salmonella</i> results	p53 induction	SHE cell results	<i>In vivo</i> results <sup>a</sup>
1	Anthraquinone	84-65-1	+	NT	—	
2	Chloroprene	126-99-8	—	NT	NT	+/+
3	1-Chloro-2-propanol	127-00-4	+	—	+	—/—
4	Cinnamaldehyde	104-55-2	+w	—	NT	
5	Citral	5392-40-5	—	+	NT	
6	Cobalt sulfate heptahydrate	10026-24-1	+w	+	+	+/+
7	Codeine	76-57-3	—	—	—	—/—
8	D&C Yellow No. 11	8003-22-3	+w	+	+	+/NT
9	Diethanolamine	111-42-2	—	—	+	+/—
10	1,2-Dihydro-2,2,4-trimethylquinoline	147-47-7	—	—	+	+/—
11	Emodin	518-82-1	+	Possibly +	+	
12	Ethylbenzene	100-41-4	—	—	+	+/+
13	Ethylene glycol monobutyl ether	111-76-2	—	—	+	+/—
14	Furfuryl alcohol	98-00-0	—	—	—	+/+
15	Gallium arsenide	1303-00-0	—	—	+	
16	Isobutene	115-11-7	—	NT	NT	+/—
17	Isobutyraldehyde	78-84-2	?	—	—	—/—
18	Methyleugenol	98-15-5	—	—	+	+/+
19	Molybdenum trioxide	1313-27-5	—	NT	+	+/—
20	Nitromethane	75-52-5	—	—	+	+/+
21	Oxymetholone	434-07-1	—	+	+	+/NT
22	Phenolphthalein	77-09-8	—	Possibly +	+	+/+
23	Primaclone	125-33-7	+	—	—	+/—
24	Pyridine	110-86-1	—	—	—	+/+
25	Scopolamine hydrobromide trihydrate	6533-68-2	—	—	+	—/—
26	Sodium nitrite	7632-00-0	+	—	+	
27	Sodium xylenesulfonate	1300-72-7	—	Possibly +	—	—/—
28	<i>t</i> -Butylhydroquinone	1948-33-0	—	+	—	—/—
29	Tetrahydrofuran	109-99-9	—	—	—	+/+
30	Vanadium pentoxide	1314-62-1	—	NT	+	

Abbreviations: +, positive; —, negative; CAS, Chemical Abstracts Registry; NT, not tested; PTE2, predictive toxicology project 2; SHE, Syrian hamster embryo; w, weak.

<sup>a</sup>Either species/transspecies.

Animal results showed equivocal results in male rats and clear evidence in female rats. Mice were not tested.

**Phenolphthalein.** A 10-mg/mL stock of phenolphthalein was prepared in DMSO, and cells were treated with doses of 1, 10, 20, 40, and 50  $\mu$ g/mL. Cells treated with phenolphthalein did not display cytotoxicity at any of the times or doses tested, and phenolphthalein induced p53 weakly by 17 hr. Phenolphthalein is therefore classified as possibly positive. This is in contrast to a negative result from the *Salmonella* assay, and in agreement with a positive result from the SHE assay. The animal results list clear evidence for carcinogenicity in male rats, male mice, and female mice, and some evidence in female rats.

**Primaclone.** A 10-mg/mL stock of primaclone was prepared in DMSO, and cells were treated with doses of 1, 10, 20, and 50  $\mu$ g/mL. Cells treated with primaclone did not display cytotoxicity at any of the times or doses tested, and primaclone did not induce p53 at the doses and times tested. Primaclone is therefore classified as negative. This is in contrast with a positive result from the *Salmonella* assay, but in agreement with a negative result from the SHE assay. The animal results list equivocal evidence for carcinogenicity in male rats, no evidence in female rats, and clear evidence in both male and female mice.

**Pyridine.** A 10-mg/mL stock of pyridine was prepared in MEM 10, and cells were treated with doses of 1, 5, 10, 25, and 50  $\mu$ g/mL. Cells treated with pyridine did not display cytotoxicity at any of the times or doses tested, and pyridine did not induce p53 at any of the doses or times tested. Pyridine is therefore classified as negative. This is in agreement with negative results from the *Salmonella* and SHE assays. The animal results list some evidence for carcinogenicity in male rats, equivocal evidence in female rats, and clear evidence for both male and female mice.

**Scopolamine hydrobromide trihydrate.** A 10-mg/mL stock of scopolamine hydrobromide trihydrate was prepared in DMSO, and cells were treated with doses of 1, 5, 10, 25, and 50  $\mu$ g/mL. Cells treated with scopolamine hydrobromide trihydrate did not display cytotoxicity at any of the times or doses tested, and scopolamine hydrobromide trihydrate did not induce p53 at any of the doses or times tested. Scopolamine hydrobromide trihydrate is therefore classified as negative. This is in agreement with a negative result from the *Salmonella* assay and contrasts with a positive SHE result. The animal results list no evidence for carcinogenicity.

**Sodium nitrate.** A 10-mg/mL stock of sodium nitrate was prepared in DMSO, and

cells were treated with doses of 1, 5, 10, 25, and 50  $\mu$ g/mL. Cells treated with sodium nitrate did not display cytotoxicity at any of the times or doses tested, and sodium nitrate did not induce p53 at any of the times or doses tested. Sodium nitrate is therefore classified as negative. The *Salmonella* and SHE results were positive.

**Sodium xylenesulfonate.** A 10-mg/mL stock of sodium xylenesulfonate was prepared in DMSO, and cells were treated with doses of 1, 5, 10, 25, and 50  $\mu$ g/mL. Cells treated with sodium xylenesulfonate did not display cytotoxicity at any of the times or doses tested, and sodium xylenesulfonate induced p53 weakly by 17 hr. Sodium xylenesulfonate is therefore classified as possibly positive. The *Salmonella* and SHE results were negative, and no evidence of carcinogenicity was observed in male or female rats or male or female mice.

***t*-Butylhydroquinone.** A 10-mg/mL stock of 5-butylhydroquinone was prepared in DMSO, and cells were treated with doses of 1, 5, 10, 25, and 50  $\mu$ g/mL. Cells treated with *t*-butylhydroquinone displayed cytotoxicity at 25 and 50  $\mu$ g/mL by 17 hr, and *t*-butylhydroquinone induced p53 by 17 hr. *t*-Butylhydroquinone is therefore classified as positive. This contrasts with negative *Salmonella* and SHE assay results. No evidence of carcinogenicity was observed in male or female rats or male or female mice.

**Tetrahydrofuran.** A 10-mg/mL stock of tetrahydrofuran was prepared in DMSO, and cells were treated with doses of 1, 5, 10, 20, 25, 50, and 100  $\mu$ g/mL. Cells treated with tetrahydrofuran did not display cytotoxicity at any of the times or doses tested, and did not induce p53 at any of the times or doses tested. Tetrahydrofuran is therefore classified as negative. The *Salmonella* and SHE results were negative. In animal studies, some evidence for carcinogenicity was found in male rats, no evidence in female rats and male mice, and clear evidence in female mice.

## Discussion

The purpose of this study was to determine the ability of the newly developed p53-induction assay to identify genotoxic substances and to predict carcinogenicity in rodents. To this end, we subjected 25 of the 30 PTE2 substances to this test. Five substances strongly and reproducibly increased cellular p53 levels and therefore were classified as positive, three substances increased cellular p53 levels, but to a less significant extent and therefore were identified as possibly positive, and seventeen substances did not significantly increase cellular p53 levels and were therefore classified as negative.

Five of the PTE2 substances were not tested. As currently configured, our assay

system is capable of testing solid or liquid substances that can be dissolved in culture media to the required concentrations and which will remain in solution for several hr at 37°C. Anthraquinone, molybdenum trioxide, and vanadium pentoxide were not tested because of the limited solubility of these substances in water, culture media, and DMSO. It may be possible in the future to identify conditions that will allow us to deliver the substances to the cells at the required concentrations. Isobutene is a gas at room temperature and was not tested; it may be possible in the future to set up a delivery system that would deliver a constant concentration of the gas to cells in culture. Chloroprene was not tested because it cannot be shipped.

The level of p53 detected in untreated cells varied between experiments from a low of undetectable (0) to a high of approximately 10 ng p53/mg total cellular protein. Therefore, each experiment was analyzed separately and the results of replicate experiments compared with each other. We found good agreement between experiments; for most chemicals, all experiments demonstrated the same increase or lack of increase, and with the same or a similar statistical significance. For a few chemicals, one experiment of three or four either failed to show the same trend, or did show the same trend but at a lower level of significance.

In this study we identified chemicals that induced p53 but to a lesser extent and to a lower level of significance as possibly positive. It may be that weakly positive would be a more appropriate classification. To distinguish between the two, it may be helpful to perform additional trials of these substances to confirm the reproducibility of the observed increases.

The strength of this p53-induction assay is expected to be in identifying genotoxic compounds, of which this PTE2 group contains only a few. We therefore drew comparisons between results from the p53-induction assay and the *in vivo* results for both the entire set of tested compounds and separately for the genotoxic substances. Figure 4 summarizes the comparisons between the *Salmonella*, p53 induction, SHE, and *in vivo* test results. Figure 4A compares the overall *in vivo* test results with each of the three *in vitro* protocols separately, and with a combination of predictions from the three *in vitro* protocols. In this analysis, chemicals were classified as *in vivo* positives if some or clear evidence of carcinogenicity was obtained in either or both species. Chemicals were classified as *in vitro* positives if they tested either positive, possibly positive, or weakly positive. Analyses included only those chemicals where both assays have been done; or, in the case of the "any of the three" analysis, all four assays have been done.



This accounts for the differences in the totals for the included analyses. Also, for the “any of the three” analysis, a chemical is scored as *in vitro* positive if any of the three tests (*Salmonella*, p53 induction, or SHE) gave a positive result. By these criteria, the *Salmonella* test gave a concordance with the *in vivo* test results of 32%, the p53-induction assay a concordance of 40%, the “any of the three” results a concordance of 59%, and the SHE assay a concordance of 67%. Therefore, by this analysis, the SHE test yielded the highest concordance with the *in vivo* results, followed by the composite, then the p53 induction, and finally the *Salmonella* assays. These results also reflect the higher likelihood of the SHE test to produce positive results for this set of chemicals, most of which were also positive in *in vivo* testing.

A comparison of the result from the p53-induction assay and the *in vivo* results for only the genotoxic compounds in this group (those that tested positive in the *Salmonella* assay) shows that two substances were positive in both (cobalt sulfate heptahydrate and D&C Yellow No. 11), one substance was positive *in vivo* but negative in the p53-induction assay (primaclone), and one substance was negative in both assays (1-chloro-2-propanol). Of this limited number of genotoxic compounds present in the PTE2 list, the p53-induction assay has a concordance of 75%.

Because the primary motivation in performing these types of studies is the protection of human health, transspecies carcinogens may be of greater concern than single-species carcinogens. Figure 4B compares the *in vivo* test results for trans-species carcinogens with each of the three *in vitro* protocols separately, and with a combination of predictions from the three *in vitro* protocols. In this analysis, chemicals were classified as *in vivo* positives only if some or clear evidence of carcinogenicity was obtained in both tested species; otherwise, the analysis was as described for Figure 4A. By these criteria, the “any of the three” results gave a concordance of 35%, the *Salmonella* test a concordance of 50%, the SHE assay a concordance of 53%, and the p53-induction assay a concordance of 56%. Therefore, by this analysis, the p53-induction assay yielded the highest concordance with the *in vivo* results, followed by the SHE analysis, then the *Salmonella*, and finally the composite assays. Because fewer of the *in vivo* results are classified as positive by the transspecies criteria, the concordance of the two assays less likely to test positive for this set of chemicals (*Salmonella* and p53 induction) with the *in vivo* test results was improved, and the concordance of the two data sets more likely to test positive for this set of chemicals (SHE and “any of the three”) decreased.

A comparison of the p53-induction assay results and the transspecies *in vivo* results for only the genotoxic compounds in this group (those that tested positive in the *Salmonella* assay) shows that one substance was positive in both assays (cobalt sulfate heptahydrate) and two substances were negative in both assays (1-chloro-2-propanol and primaclone). Of this limited number of genotoxic compounds present in the PTE2 list, the p53-induction assay has a concordance with the *in vivo* results of 100%.

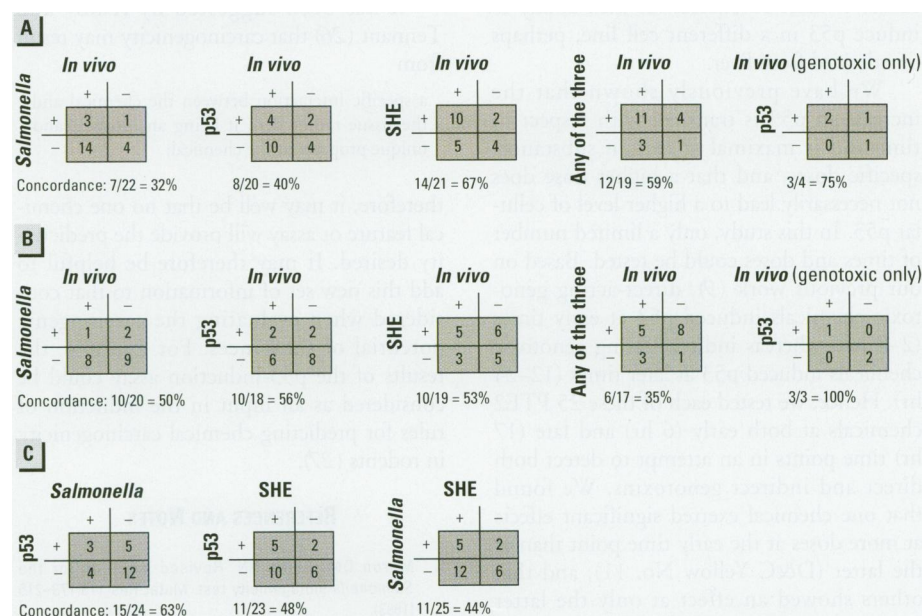
Figure 4C compares results from the three *in vitro* assays with each other. In this analysis, only those chemicals for which both listed assays have been reported are included. The concordance of *Salmonella* and SHE assays is 44%, that of the p53-induction and SHE assays is 48%, and the p53-induction and *Salmonella* assays is 63%, indicating that of the three, the p53 induction and the *Salmonella* assays are the most like each other, although still significantly different.

Based on the biology underlying the *Salmonella*, SHE, and p53-induction assays, it seems clear that the p53-induction assay senses a fundamentally different type of biological information than do the other methods. The Ames test (1) is perhaps the most widely used test for genotoxicity; it is based on the ability

of mutagens to alter the phenotype of specific strains of *Salmonella* by inducing heritable changes in its genetic material. The SHE test likewise relies on heritable changes in the morphology of treated cells (22). In contrast, the p53-induction assay takes advantage of a normal cellular response to DNA damage; namely, the increase of the tumor-suppressor protein p53. It is therefore not dependent on a permanent change in DNA; rather, it works by eavesdropping on the events that normally follow DNA damage.

An advantage of this assay, therefore, is that it may be capable of detecting transitory DNA damage that is repaired too quickly to be detected in tests which require heritable changes in the DNA. Diethylstilbestrol, which produces DNA lesions with a short biologic half-life, induced p53 [(9) and references therein] while giving a negative or weak response in the *Salmonella* assay. This feature is one possible explanation for substances (such as sodium xylenesulfate or *t*-butylhydroquinone) testing positive or possibly positive in this assay while testing negative in the *in vivo* tests; the substance may have caused DNA damage that was quickly repaired before DNA replication.

Another possible explanation for chemicals testing positive in this assay and negative



**Figure 4.** Comparisons of assay results. Abbreviations: +, positive; -, negative; SHE, Syrian hamster embryo. (A) *In vitro* results as compared to *in vivo* results (single- and transspecies carcinogens combined). The results of the three *in vitro* assays (*Salmonella*, p53 induction, and SHE) were compared against the *in vivo* results. Chemicals were classified as *in vivo* positives if some or clear evidence of carcinogenicity was obtained in either or both species. For the last panel, only *Salmonella* positive chemicals were included in the analysis. (B) *In vitro* results compared against *in vivo* results (transspecies carcinogens only). The results of the three *in vitro* assays were compared against the *in vivo* results. In this case, chemicals were classified as *in vivo* positives only if some or clear evidence of carcinogenicity was obtained in both tested species. For the last panel, only *Salmonella* positive chemicals were included in the analysis. (C) Results from the three *in vitro* assays as compared to each other. For the “any of the three” analyses in (A) and (B), a chemical is scored as *in vitro* positive if any of the three tests yielded a positive result. Also, two chemicals (D&C Yellow No. 11 and oxymetholone) were tested only in one species and are therefore included in the analysis in (A) but not in (B).

in the *in vivo* protocol is that they could cause increases in cellular p53 levels by a mechanism independent of DNA damage (23). For example, a substance that could block proteasome function would be expected to increase cellular p53 (24,25). We have found, however, that the ability of substances to increase p53 and to cause cellular cytotoxicity is not correlated; indeed, most substances do not induce p53 even when clearly inducing stress and cytotoxicity [(9) and this study]. Hence, it seems unlikely that p53 induction is a general nonspecific response to chemical or cytotoxic stress.

The p53-induction assay used in this study utilized NCTC 929 cells, which are a mouse fibroblast cell line. Although we have shown that these cells can respond to indirect genotoxins such as aflatoxin B<sub>1</sub> by increasing their level of p53, demonstrating the presence of activating metabolic enzymes, it is possible that differences in the array of metabolic activities in these cells as opposed to liver extract could create a different combination of metabolites. This altered range of activities could then either increase or decrease DNA damage. This could account for differences in the results noted with the p53-induction assay and those noted for other assays. It may be interesting to test a subset of these substances for their ability to induce p53 in a different cell line, perhaps one derived from liver.

We have previously shown that the increase in p53 is transient with respect to time and is maximal at certain substance-specific doses, and that a higher dose does not necessarily lead to a higher level of cellular p53. In this study, only a limited number of times and doses could be tested. Based on our previous work (9), direct-acting genotoxic chemicals induced p53 at early times (2–8 hr), whereas indirect-acting genotoxic chemicals induced p53 at later times (12–24 hr). Hence, we tested each of these 25 PTE2 chemicals at both early (6 hr) and late (17 hr) time points in an attempt to detect both direct and indirect genotoxins. We found that one chemical exerted significant effects at more doses at the early time point than at the latter (D&C Yellow No. 11), and that others showed an effect at only the latter time point (citral, oxymetholone, *t*-butylhydroquinone; compare Figure 2 and Figure 1). It is possible, however, that a substance capable of weakly inducing p53 could exert its maximal effect at a time other than those tested, and that any induction seen at the tested times was not statistically significant.

Previously tested genotoxins yielded maximal induction at doses between 1 and 100 µg/mL; therefore, for each of the 25 substances analyzed, we tested several doses

between 0.5 and 100 µg/mL. We found that most of the chemicals that tested positive did so only at one or a few of the tested doses, suggesting that the dose range at which an effect can be observed may be rather narrow. For a number of the substances, the higher doses (25, 50, and 100 µg/mL) induced extensive cytotoxicity, compromising the validity of the p53 assay at those dosages. It is possible that a substance testing negative in our assay would have tested positive at some other untested dose. Our results were analyzed with the Scheffe *S*-test, which is a relatively conservative post hoc test for differences.

The expected strength of this p53-induction assay is in the identification of genotoxic compounds. There were few of these compounds in this PTE2 group, and with these few compounds, the p53-induction assay did an excellent job of identifying them. The most likely explanation for the inability of this assay to identify many of the carcinogenic compounds in this group is that they are epigenetic substances that cause cancer by nongenotoxic mechanisms. Additionally, factors such as absorption, distribution, metabolism, and excretion, which operate in whole animals but do not operate in *in vitro* test systems, could play a role.

It has been suggested by Ashby and Tennant (26) that carcinogenicity may result from

a specific interaction between the chemical and the tissue rather than it being an intrinsic and unique property of the chemical;

therefore, it may well be that no one chemical feature or assay will provide the predictivity desired. It may therefore be helpful to add this new set of information to that considered when evaluating the carcinogenic potential of substances. For example, the results of the p53-induction assay could be considered as an input in the induction of rules for predicting chemical carcinogenicity in rodents (27).

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